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All plakin family proteins are known to be autoantigens in paraneoplastic pemphigus (PNP). In this study, we first examined whether PNP sera also react with epiplakin, another plakin protein, by various immunological methods using 48 Japanese PNP sera. Immunofluorescence confirmed that cultured keratinocytes expressed epiplakin. Epiplakin was detected by 72.9% of PNP sera by immunoprecipitation-immunoblotting with KU-8 cell extract, but not by immunoblotting of either normal human epidermal extract or KU-8 cell extract. Epiplakin was essentially not detected by 95 disease and normal control sera. Statistical analyses of various clinical and immunological findings revealed a significant correlation of the presence of anti-epiplakin antibodies with both bronchiolitis obliterans and mortality. No epiplakin-negative PNP case developed bronchiolitis obliterans. However, although 29.4% of European patients with PNP had bronchiolitis obliterans, significant correlation with anti-epiplakin autoantibodies was not observed. In further studies for lung, immunofluorescence showed the presence of epiplakin in normal human lung, particularly respiratory bronchiole, immunoprecipitation-immunoblotting showed that PNP sera reacted with epiplakin in cultured lung cells, and mice injected with polyclonal antibody specific to epiplakin histopathologically showed abnormal changes in small airway epithelia. These results indicated that epiplakin is one of the major PNP autoantigens and is related to PNP-related bronchiolitis obliterans.

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INTRODUCTION

Paraneoplastic pemphigus (PNP), or paraneoplastic autoimmune multiorgan syndrome (Nguyen et al., 2001), is an autoimmune blistering skin disease with severe mucocutaneous lesions. PNP is commonly associated with hematological malignancies (Anhalt, 1997; Anhalt et al., 1990). Histopathology shows acantholysis and apoptotic cells in epidermis and interface changes (Anhalt, 1997; Anhalt et al., 1990; Billet et al., 2006; Sehgal et al., 2009).

Prognosis of PNP is poor with approximately 68% mortality (Leger et al., 2012). Particularly, all PNP cases with chronic respiratory disease show fatal outcome (Nousari et al., 1999; Takahashi et al., 2000). Severe inflammation occurring in respiratory bronchioles leads to irreversible fibrotic reaction, resembling bronchiolitis obliterans (BO). In a previous study, BO occurred in 6% of 53 European patients with PNP (Leger et al., 2012), whereas the prevalence was 25% in our recent study for 107 Japanese patients with PNP. However, pathogenesis in PNP-related BO is currently unknown.

PNP develops autoantibodies to various antigens, mainly plakin family proteins. Immunoprecipitation (IP) first detected the 250-kDa desmoplakin I, the 230-kDa bullous pemphigoid 230 (BP230), the 210-kDa doublet of desmoplakin II and unknown protein, and the 190-kDa and 170-kDa unknown proteins (Anhalt et al., 1990). Then, immunoblotting (IB) of normal human epidermal extract showed that all PNP sera reacted with the 210-kDa and 190-kDa doublet proteins (Borradori et al., 1998; Hashimoto, 2001; Hashimoto et al., 1995). Thereafter, the 210-kDa and 190-kDa proteins were identified as envoplakin (EPL) and periplakin (PPL), respectively (Kiyokawa et al., 1998). This reactivity is very useful for

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Abbreviations: BO, bronchiolitis obliterans; BP, bullous pemphigoid; Dsg, desmoglein; EPL, envoplakin; EPPK, epiplakin; IB, immunoblotting; IF, immunofluorescence; IHC, immunohistochemistry; IP, immunoprecipitation; NHSAE, normal human small airway epithelial; pAb, polyclonal antibody; PNP, paraneoplastic pemphigus; PPL, periplakin; RP, recombinant protein

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diagnosis of PNP (Joly et al., 2000; Mouquet et al., 2008; Poot et al., 2013). Plectin, another plakin protein, was also found to be PNP autoantigen (Proby et al., 1999). Thus, all known plakin proteins are PNP autoantigens.

Non-plakin proteins are also detected by PNP sera. ELISAs detected antibodies to desmoglein 3 (Dsg3) and/or Dsg1 in PNP sera (Amagai et al., 1998; Brandt et al., 2012). We also found that the unknown 170-kDa protein was alpha-2-macroglobulin-like-1 (Numata et al., 2013; Schepens et al., 2010), and BP180 was frequently reacted by PNP sera (Tsuchisaka et al., 2014).

Epiplakin (EPPK) was originally identified as an autoantigen in a patient (Fujiwara et al., 1996), and is expressed in entire epidermis, skin appendices, and other intestinal epithelia (Fujiwara et al., 2001). Subsequent studies showed that EPPK is a plakin protein with many repeats of plakin-specific B-domain and linker-domain (Spazierer et al., 2003; Takeo et al., 2003). EPPK connects intermediate filaments (Spazierer et al., 2006, 2008; Wang et al., 2006). Despite abundant expression of EPPK in stratified and simple epithelia, gene-targeted mice showed no abnormality in either phenotype or keratin filament organization, except for mild delay of wound healing (Goto et al., 2006, Ishikawa et al., 2010).

Because EPPK is a plakin protein, we speculated that EPPK might also be a PNP autoantigen. In this study, we investigated whether anti-EPPK antibodies were present in PNP sera by various analyses. Immunoprecipitation-immunoblotting (IP-IB) detected anti-EPPK autoantibodies in 35 of 48 PNP sera. Statistical analysis indicated correlations of anti-EPPK antibodies with PNP-related BO and mortality. Additional studies indicated that EPPK is expressed in respiratory cells and tissues and was reacted by PNP sera, suggesting that anti-EPPK autoantibodies may develop BO.

RESULTS

Clinical and immunological parameters for all 48 Japanese patients with PNP are summarized in Supplementary Table S1 online.

Immunohistochemistry (IHC) and immunofluorescence (IF) of normal human skin, KU-8 cells, and rat bladder

By IHC and IF of normal human skin, rabbit polyclonal antibody (pAb) for human EPPK (anti-EPPK pAb) (Figure 1a and c), but not normal rabbit IgG (Figure 1b and d), showed cytoplasmic staining in entire epidermis. By IF, anti-EPPK pAb (Figure 1e), but not normal rabbit IgG (Figure 1f), showed perinuclear cytoplasmic staining in cultured KU-8 cells. Finally, by IF using the rat bladder, anti-EPPK pAb (Figure 1g), but not normal rabbit IgG (Figure 1h), showed cell surface and cytoplasmic reactivity.

IB of normal human epidermal extract

We first attempted to detect anti-EPPK antibodies in 48 Japanese PNP sera by IB of normal human epidermal extract. Anti-EPPK pAb detected approximately 500-kDa EPPK (Supplementary Figure S1 online). However, whereas all PNP sera detected the 210-kDa EPL and 190-kDa PPL, neither PNP nor normal sera reacted with EPPK.

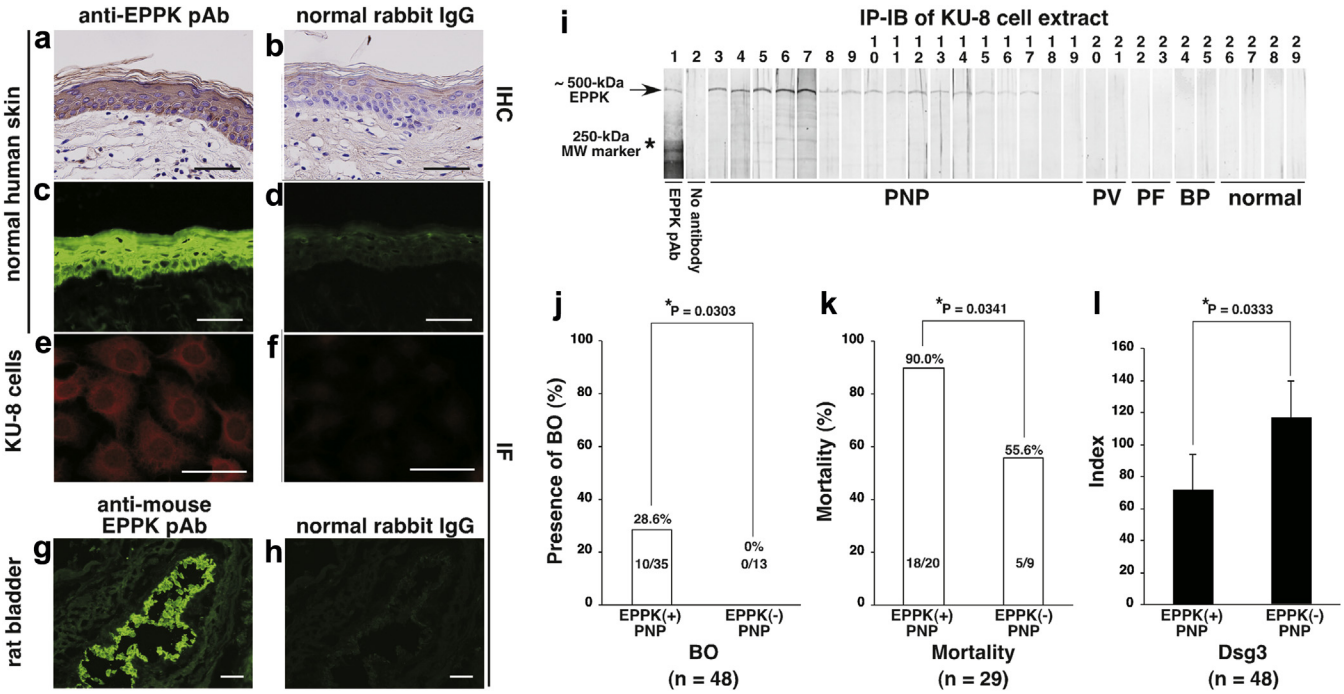

Figure 1. EPPK detection in normal human skin and KU-8 cells. (**a, b**) IHC of normal human skin with anti-EPPK pAb and normal rabbit IgG. (**c**–**h**) IF of normal human skin (**c, d**), KU-8 cells (**e, f**), and rat bladder (**g, h**). Bars = 50 μm. (**i**) IP-IB of KU-8 cell extract for EPPK with anti-EPPK pAb (lane 1), no antibody (lane 2), PNP (lanes 3–19), PV (lanes 20, 21), PF (lanes 22, 23), BP (lanes 24, 25), and normal sera (lanes 26–29). The positions of the approximately 500-kDa EPPK and the 250-kDa molecular marker are shown on the left. (**j**–**l**) Statistical analyses for association of BO (**j**), mortality (**k**), and ELISA indices for Dsg3 (**l**). Asterisks (*) indicate statistically significant differences between EPPK(+) PNP and EPPK(−) PNP ($P < 0.05$). BO, bronchiolitis obliterans; BP, bullous pemphigoid; Dsg, desmoglein; EPPK, epiplakin; IF, immunofluorescence; IHC, immunohistochemistry; IP-IB immunoprecipitation-immunoblotting; pAb, polyclonal antibody; PNP, paraneoplastic pemphigus; PV, pemphigus vulgaris.

Absorption analysis of anti-EPPK pAb with EPPK recombinant protein (RP)

In IB of normal human epidermal extract, anti-EPPK pAb also reacted with many lower molecular weight protein bands, which may be other molecules or degraded EPPK fragments. To confirm the specificity of anti-EPPK pAb, we performed absorption analysis using human EPPK RP (Supplementary Figure S2a online). Both intact EPPK and additional lower protein bands seen before absorption were significantly reduced by absorption with 0.3 μg EPPK RP, and completely disappeared with 0.6 and 1.2 μg EPPK RP. The reduction of EPPK reactivity was not seen by absorption with 5 and 10 μg of total protein in lysate of *Escherichia coli* transformed with the pGEX empty vector.

IB of KU-8 cell extract

Because we confirmed strong expression of EPPK in KU-8 cells by IF, we then tried to detect anti-EPPK antibodies in PNP sera by IB using KU-8 cell extract. Anti-EPPK pAb strongly reacted with the approximately 500-kDa EPPK, as well as additional lower bands (Supplementary Figure S3 online). However, none of the representative PNP sera reacted with EPPK, although all sera reacted with EPL and PPL. No normal sera reacted with any plakin proteins.

IP-IB of KU-8 cell extract

To overcome the difficulty in detection of EPPK by IB analyses, we performed IP-IB of KU-8 cell extract (Figure 1i for representative sera, and Supplementary Figure S4 online for all sera). Nineteen PNP sera and anti-EPPK pAb reacted strongly and exclusively with the approximately 500-kDa EPPK, 16 PNP sera showed relatively weak but clear reactivity with EPPK, and 13 PNP sera showed no reactivity. Thus, 35 (72.9%) of 48 PNP sera reacted with EPPK. This EPPK band was not detected by 20 pemphigus vulgaris and 20 pemphigus foliaceus sera, but was very weakly detected by one of 20 BP and two of 35 normal sera (Supplementary Figure S4).

We also performed this IP-IB for additional 22 European PNP sera. Eight sera reacted strongly and exclusively with EPPK, and four sera reacted relatively weakly with EPPK (Supplementary Figure S5 and Table S2 online). Thus, European PNP sera also clearly and frequently reacted with EPPK.

IP-IB of KU-8 cell extract preabsorbed with anti-EPPK pAb

To further confirm the specific reactivity of PNP sera with EPPK, we performed IP-IB using KU-8 cell extract, which was preabsorbed with anti EPPK pAb. In this IP-IB, two selected PNP sera reacted with EPPK in non-preabsorbed KU-8 cell extract, but not in KU-8 cell extract preabsorbed with anti-EPPK pAb (Supplementary Figure S6 online).

Combined IP-IB of IP with anti-EPPK pAb and IB with antibodies specific to various plakin family proteins

To further confirm the lack of cross-reactivity with the anti-EPPK pAb with other plakin proteins, after IP of KU-8 extract with anti-EPPK pAb, immunoprecipitated proteins were immunoblotted with antibodies specific to various plakin proteins. As expected, EPPK was detected strongly in both KU-8 extracts and immunoprecipitates (Supplementary Figure S7a online). EPL and PPL were detected in KU-8 extracts but not in immunoprecipitates. Plectin and desmoplakin were not detected in both extracts of KU-8 cells and immunoprecipitated samples, suggesting that antibodies to these plakins were not suitable for IB. Nevertheless, the results for EPL and PPL strongly indicated that anti-EPPK pAb had no cross-reactivity with other plakins.

IF of mouse skin and lung

To further demonstrate the specificity of anti-EPPK pAb and anti—mouse-EPPK pAb, we performed IF of skin and lung tissues of wild-type and *Eppk*-knockout mice using these pAbs. Both pAbs reacted with wild-type mouse tissues, but no positive reactivity was observed in *Eppk*-knockout mouse tissues (Supplementary Figure S7b and c). These results indicated that both pAbs were specific to EPPK in mouse tissues, and had no cross-reactivity with other plakins.

IB of purified EPPK RP

We also performed IB of EPPK RP for 48 PNP sera. Both anti-GST monoclonal antibody and anti-EPPK pAb reacted strongly with the 80-kDa EPPK RP, as well as additional lower bands that were considered degradation products (Supplementary Figure S8 online). The EPPK RP was also recognized weakly by almost all PNP sera, including sera negative for EPPK in IP-IB. None of control pemphigus vulgaris, pemphigus foliaceus, BP, and normal sera reacted with the RP.

Statistical analysis between clinical and immunological features and EPPK reactivity in 48 Japanese patients with PNP

We designated 35 EPPK-positive Japanese PNP cases and 13 EPPK-negative PNP cases in IP-IB as EPPK(+) PNP and EPPK(−) PNP, respectively. Relevant results in statistical analyses are graphically shown (Figure 1j—l). EPPK(+) PNP showed significantly higher frequency of BO than EPPK(−) PNP ($P = 0.0303$), and none of the 13 patients with EPPK(−) PNP had BO (Figure 1j). Mortality was significantly higher in EPPK(+) PNP ($P = 0.0341$) (Figure 1k). Dsg3 indices in EPPK(+) PNP (71.47 ± 66.93) were significantly lower than EPPK(−) PNP (116.57 ± 90.80) ($P = 0.0333$) (Figure 1l). The results of other parameters showed no statistical significance between EPPK(+) PNP and EPPK(−) PNP (Table 1).

We also obtained information of BO in 17 of 22 European patients with PNP (Supplementary Table S2).However, there was no significant relationship between anti-EPPK antibodies and BO in European patients with PNP (Mann-Whitney *U*-test; $P = 0.297$).

IHC and IF of normal human lung tissue and cultured lung cells

Because statistical analyses showed a clear relationship between EPPK(+) PNP and BO, we then examined whether EPPK is present in lung tissues and respiratory cells. By IHC and IF of normal human lung samples obtained from three unrelated subjects, anti-EPPK pAb reacted relatively weakly with alveolus cells (Figure 2a and c) and strongly with bronchiole epithelia (Figure 2a, b, d, and e). Particularly, granular staining was observed inside bronchiole epithelial cells (Figure 2e, arrows). Normal rabbit IgG did not react with these cells (Figure 2g, h, i, j, and k).

By IF of cultured normal human small airway epithelial (NHSAE) cells, anti-EPPK pAb showed perinuclear

Table 1. Results of clinical and immunological features in 48 patients with PNP and differences between EPPK(+) PNP and EPPK(−) PNP, which were not statistically significant ($P > 0.05$), except for BO ($P = 0.0303$)

Parameters (n = cases with description)	EPPK(+) PNP	EPPK(−) PNP	Total
Age (n = 46)	59.09 ± 11.97	53.77 ± 13.48	57.59 ± 12.49
Gender (n = 47)			
Females	24/34 (70.6%)	9/13 (69.2%)	33/47
Males	10/34 (29.4%)	4/13 (30.8%)	14/47
Cutaneous lesions			
Trunk (n = 44)	20/33 (60.6%)	9/11 (81.8%)	29/44
Extremities (n = 44)	18/33 (54.5%)	5/11 (45.5%)	23/44
Mucous lesions			
Oral (n = 44)	33/33 (100.0%)	11/11 (100.0%)	44/44
Ocular (n = 39)	16/28 (57.1%)	8/11 (72.7%)	24/39
Nasal (n = 39)	8/28 (28.6%)	2/11 (18.2%)	10/39
Genital (n = 37)	10/27 (37.0%)	6/10 (60.0%)	16/37
BO (n = 48)	10/35 (28.6%)	0/13 (0%)	10/48
Acantholysis (n = 39)	14/29 (48.3%)	8/10 (80.0%)	22/39
Necrotic cells (n = 39)	9/29 (31.0%)	5/10 (50.0%)	14/39
Liquefaction degeneration (n = 39)	5/29 (17.2%)	1/10 (10.0%)	6/39
Reactivity for therapy (n = 43)	13/31 (41.9%)	9/12 (75.0%)	22/43
Rat bladder indirect IF (n = 48)	27/35 (77.1%)	9/13 (69.2%)	36/48
Human skin indirect IF (n = 48)	22/35 (62.9%)	11/13 (84.6%)	33/48
Dsg1 (n = 48)	23.94 ± 40.28	14.98 ± 20.38	21.51 ± 36.00

Abbreviations: BO, bronchiolitis obliterans; Dsg, desmoglein; EPPK, epiplakin; IF, immunofluorescence; PNP, paraneoplastic pemphigus.

cytoplasmic staining (Figure 2f), similar to that seen in KU-8 cells (Figure 1e). Normal rabbit IgG showed no staining (Figure 2l).

IB of NHSAE cell extract

By IB of NHSAE cell extract, anti-EPPK pAb reacted strongly with the approximately 500-kDa EPPK, as well as smear-like additional lower bands (Figure 3a). However, none of representative PNP sera with or without BO showed the reactivity with EPPK (Figure 3a). PNP sera reacted with PPL but not EPL. Normal sera reacted with no plakin protein in NHSAE cell extract.

Interestingly, whereas KU-8 cell extract showed only one EPPK protein band (K-EPPK for keratinocyte-EPPK), NHSAE cell extract showed two different EPPK bands, K-EPPK and L-EPPK (for lung-EPPK), with weaker reactivity with L-EPPK (Figure 3b).

IP-IB of NHSAE cell extract

We next performed IP-IB of NHSAE cell extract for representative BO(+) and BO(−) PNP sera (Figure 3c). Anti-EPPK pAb reacted with doublet bands of the approximately 500-kDa EPPK, with stronger reactivity with the lower band (K-EPPK) and weaker reactivity with the upper band (L-EPPK). Some of both BO(+) and BO(−) PNP sera reacted with the doublet EPPK bands in the same pattern. No normal sera showed this reactivity.

Injection of rabbit anti−mouse-EPPK pAb into mice

To further investigate whether EPPK relates to BO, we performed injection experiments of anti−mouse-EPPK pAb into mice. In our preliminary study, the anti−mouse-EPPK pAb reacted with the approximately 700-kDa mouse EPPK by IB of mouse skin extract, and the reactivity reduced significantly by preabsorption with mouse EPPK RP (Supplementary Figure S2b), confirming the specific reactivity with EPPK.

In injection experiments, to exclude the nonspecific effect of normal IgG injection, we first injected normal rabbit IgG at doses of 0 (control), 1, 2, 4, and 6 mg ($n = 3$) into BALB/c mice, and bred for 2 weeks. Histopathologically, no abnormal morphological changes were found in both skin and lung tissues taken from the mice (Supplementary Figure S9 online).

In preliminary IF of skin and lung tissues from noninjected mice, anti−mouse-EPPK pAb clearly stained entire epidermis and bronchiole epithelia, respectively (Figure 4a).

In mice injected with anti−mouse-EPPK pAb, direct IF of skin and lung tissues using FITC-conjugated anti-rabbit IgG showed positive staining of entire epidermis and bronchiole epithelia, respectively (Figure 4b), indicating that injected anti−mouse-EPPK pAb actually penetrated into skin and lung cells.

In mice injected with anti−mouse-EPPK pAb, the histopathological study showed no obvious abnormality in skin (Figure 4c). In contrast, lung showed mononuclear cell infiltration and extracellular matrix deposition in peribronchial areas, as well as detached epithelial cells, suggesting loss of cell-cell adhesion (Figure 4c). No significant change was observed in lung tissue from mice injected with normal rabbit IgG.

DISCUSSION

In this study, we first showed that none of 48 Japanese PNP sera reacted with EPPK by IB of both normal human epidermal extract and cultured KU-8 cell extract, probably due to loss of conformation of EPPK during IB procedure. Then, we performed IP-IB of KU-8 cell extract, which

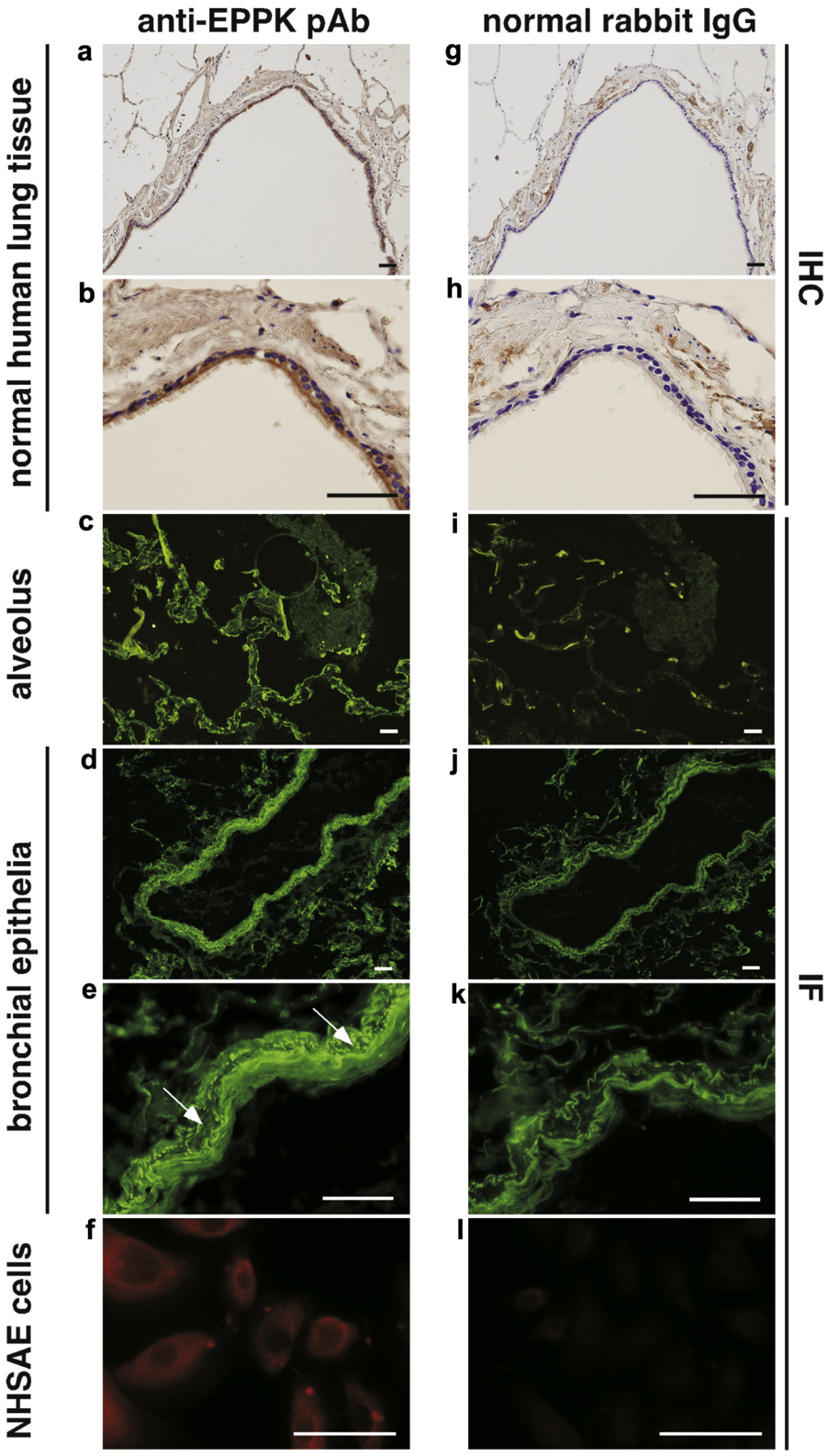

Figure 2. IHC and IF of normal human lung tissue and NHSAE cells. (**a, b, g, h**) IHC of normal human lung tissue with anti-EPPK pAb and normal rabbit IgG. (**c**–**f, i**–**l**) IF of normal human lung tissue (**c, i**: alveolus, **d, e, j, i**: bronchial epithelia) and NHSAE cells (**f, l**) with anti-EPPK pAb and normal rabbit IgG. Bars = 50 μm. EPPK, epiplakin; IF, immunofluorescence; IHC, immunohistochemistry; NHSAE, normal human small airway epithelial; pAb, polyclonal antibody.

successfully detected anti-EPPK antibodies in 35 (72.9%) of 48 PNP sera. By IHC and IF, anti-EPPK pAb showed clearly positive reactivity in both normal human skin and rat bladder, suggesting high and wide expression of EPPK in various epithelia.

Our statistical analysis revealed that BO was significantly more prevalent in EPPK(+) PNP than EPPK(−) PNP. Several mechanisms for PNP-related BO were proposed (Nousari et al., 1999; Takahashi et al., 2000). Airway mucosa in patients with PNP with BO showed acantholysis and IgG deposition in bronchial epithelium, suggesting that autoantibody-mediated acantholytic process occurred in respiratory tissues. However, because bronchial epithelia express only Dsg2, anti-Dsg1 and Dsg3 autoantibodies in

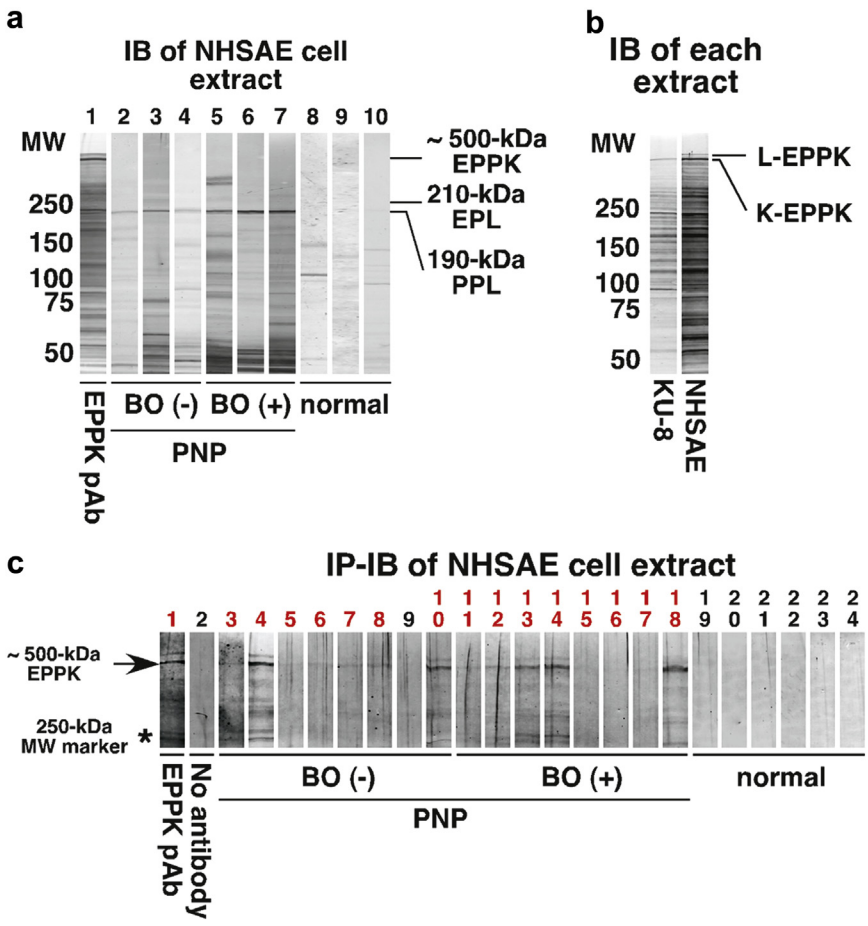

Figure 3. IB and IP-IB of NHSAE cell extract. (**a**) IB of NHSAE cell extract with anti-EPPK pAb (lane 1), BO(−) PNP (lanes 2−4), BO(+) PNP (lanes 5−7), and normal sera (lanes 8−10). (**b**) Comparative IB study of KU-8 cell and NHSAE cell extracts with anti-EPPK pAb. The positions of molecular markers are shown on the left (**a, b**). The positions of EPPK, EPL, and PPL (**a**) and those of K-EPPK and L-EPPK (**b**) are shown on the right. (**c**) IP-IB of NHSAE cell extract with anti-EPPK pAb (lane 1), no antibody (lane 2), BO(−) PNP (lanes 3−10), BO(+) PNP (lanes 11−18), and normal sera (lanes 19−24). Red numbers indicate lanes with positive reactivity with EPPK. BO, bronchiolitis obliterans; BP, bullous pemphigoid; Dsg, desmoglein; EPL, envoplakin; EPPK, epiplakin; IF, immunofluorescence; IHC, immunohistochemistry; IP-IB immunoprecipitation-immunoblotting; NHSAE, normal human small airway epithelial; pAb, polyclonal antibody; PNP, paraneoplastic pemphigus; PPL, periplakin; PV, pemphigus vulgaris.

PNP sera should not contribute to this change. In contrast, respiratory epithelium expresses various plakin proteins including EPPK. Therefore, we speculated that anti-EPPK antibodies in PNP sera may react with EPPK in lung tissues and cause BO.

To confirm this speculation, we first showed that EPPK was present in normal lung tissue and NHSAE cells by IHC and IF using anti-EPPK pAb. EPPK expressed strongly in bronchiole epithelia and weakly in alveolar cells. IB also confirmed expression of EPPK in NHSAE cells. Similar to IB of epidermal extract or KU-8 cell extract, no PNP sera recognized EPPK in IB of NHSAE cell extract. In contrast, in IP-IB of NHSAE cell extract, most sera from patients with BO(+) PNP and patients with BO(−) PNP reacted with both L-EPPK (weakly) and K-EPPK (strongly). This positive reactivity of PNP sera with EPPK in lung cells further suggested that anti-EPPK autoantibodies cause BO. However, the result that both BO(+) PNP and BO(−) PNP reacted with EPPK in NHSAE cells made the pathogenic role of EPPK in the development of BO obscure.

To further unravel the pathogenic role of EPPK in BO development, we injected anti−mouse-EPPK pAb into mice. IF indicated that the injected anti−mouse-EPPK pAb could penetrate into the skin and lung cells of mice. Histopathology revealed abnormal changes in lung tissue, but not in skin. These results further indicated that anti-EPPK antibodies can develop BO. Similar abnormal histopathological features were also shown in CXCL10 transgenic mice with BO-like inflammation (Jiang et al., 2012).

However, other mechanisms in the development of BO were also proposed. Proinflammatory cytokines and chemokines and growth factors were secreted from epithelial cells or T cells during BO development (Boehler et al., 2003). In a recent study, Dsg3 expression in lung was observed in a PNP mouse model using Dsg3-target mice and naphthalene treatment (Hata et al., 2013). Pten knockout mice showed lung fibrosis with upregulation of snail and downregulation of E-cadherin and tight junction proteins (Miyoshi et al., 2013). Therefore, these other factors and anti-EPPK antibodies may coordinately develop BO.

The mechanism by which injected pAb accessed intracytoplasmic EPPK in skin and lung was currently unclear. However, the penetration of autoantibodies into living cells was reported for antinuclear antibodies in systemic lupus erythematosus (Rekvig et al., 2012; Toubi and Shoenfeld, 2007) and for antimelanocyte antibodies in vitiligo (Ruiz-Argüelles et al., 2007). Similar mechanisms may occur for anti- EPPK antibodies.

In contrast to lung, abnormal morphology was not found in the skin of mice injected with anti−mouse-EPPK pAb. EPPK knockout mice did not show abnormal epidermis or hair (Goto et al., 2006). These findings may indicate that EPPK is not essential in the development and structural integrity of skin.

European patients with PNP were reported to show lower incidence of BO development and lower mortality (Leger et al., 2012). Therefore, we also performed IP-IB for European PNP sera and found that 54.5% of the sera reacted

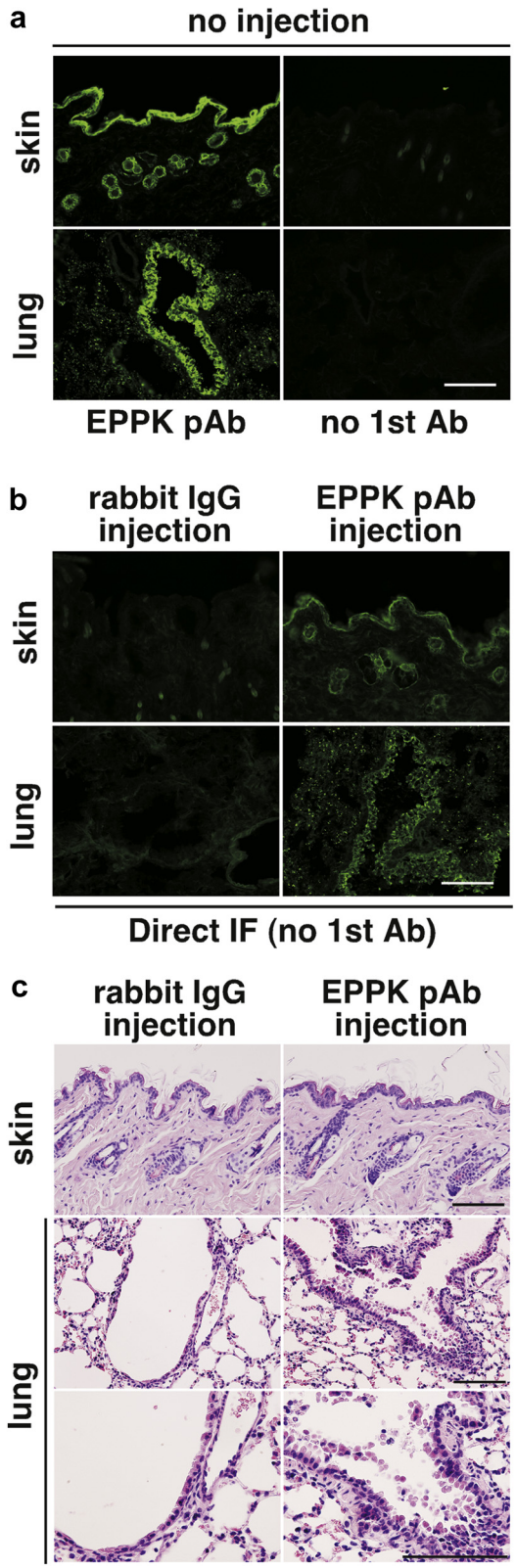

Figure 4. Injection experiments of anti−mouse-EPPK pAb into mice. (**a**) Results of IF of skin and lung tissue from noninjected mice using anti−mouse-EPPK pAb. (**b**) Results of direct IF of skin and lung tissue from mice injected with anti−mouse-EPPK pAb or normal rabbit IgG using FITC-conjugated anti-rabbit IgG without the first antibody (no 1st Ab). (**c**) Histopathological findings of skin and lung tissue from mice injected with anti−mouse-EPPK pAb or normal rabbit IgG (hematoxylin and eosin staining). Bars = 100 μm. EPPK, epiplakin; IF, immunofluorescence; pAb, polyclonal antibody.

with EPPK, suggesting that anti-EPPK antibodies are detected in PNP sera across racial lines. However, there was no significant relationship between anti-EPPK antibodies and BO in European PNP. The different results between Japanese patients with PNP and European patients with PNP might be caused by different genetic background, including HLA, although a small cohort for European PNP in our study may also explain the difference.

This study indicated the presence of two different EPPK proteins, that is, smaller K-EPPK and larger L-EPPK. Epidermal KU-8 cells expressed only K-EPPK, whereas respiratory NHSAE cells expressed both K-EPPK and L-EPPK. EPPK has phosphorylation sites, but no glycosylation sites (Dephous et al., 2008; Olsen et al., 2006; Yu et al., 2007). However, different phosphorylation cannot explain the difference in migration in the huge 500-kDa protein on SDS-PAGE. Thus, the different sizes in EPPK may be due to protein truncation by alternative splicing or proteolysis.

A recent IB study of A549 cell extract suggested the presence of anti-PPL autoantibodies in idiopathic pulmonary fibrosis (Taille et al., 2011), suggesting that autoantibodies to plakin proteins cause various inflammatory lung diseases. We also performed preliminary studies using A549 cells. However, A549 cells did not express EPPK (data not shown). This may be explained by the fact that A549 cells originated from human adenocarcinoma of alveolar basal epithelial cells (Giard et al., 1973), which expressed a very low level of EPPK. Our IB of NHSAE cells detected PPL but not EPL, whereas a previous IP study of normal human bronchiole epithelial cells detected all desmoplakin, PPL and EPL (Nousari et al., 1999). These results indicate that different respiratory cells may express plakins in different patterns. Therefore, it may also be possible to speculate that anti-EPPK autoantibodies cause idiopathic pulmonary fibrosis.

Statistic analyses also indicated that EPPK(+) PNP showed a lower level of anti-Dsg3 antibodies. Anti-Dsg3 antibodies are pathogenic in a mouse model for both pemphigus vulgaris (Amagai, 1996; Amagai et al., 1991) and PNP (Amagai et al., 1998). Thus, the lower level of anti-Dsg3 antibodies generally means lower disease activity. However, in our study, EPPK(+) PNP cases with lower anti-Dsg3 autoantibodies actually showed higher mortality, indicating that anti-EPPK antibodies may cause fatal outcome.

Intriguing issue in this study was the possibility of the cross reactivity of anti-EPPK antibodies among various plakin family proteins. EPPK has a unique structure with many repeats of plakin-specific B-domain and linker-domain, which are also present in C-terminal regions of other plakin proteins, except for PPL (Supplementary Figure S10a online). Indeed, amino acid sequence alignment revealed an identical domain of eight amino acid residues in B-domains in all plakin proteins, except for PPL (Supplementary Figure S10b).

In our IB studies, pAbs to both human and mouse-EPPKs showed additional smaller protein bands, which might be other plakin proteins. However, preabsorption of pAbs with human and mouse EPPK RPs abolished reactivity with all protein bands, indicating that the lower proteins were degradation products of EPPK and both pAbs were specific to EPPK.

In addition, we compared linker regions between 8th and 9th B-domains and between 15th and 16th B-domains, which were used to prepare pAbs to human and mouse EPPKs, respectively, among various human and mouse plakin proteins (Supplementary Table S3 online). For both the linker regions, homology between human and mouse EPPKs was more than 50%, whereas homologies between EPPK and other plakin proteins were very low. This result also suggested that both pAbs should be specific to EPPK.

Furthermore, our combined IP-IB using antibodies specific to various plakins for IB indicated that samples immunoprecipitated by anti-EPPK pAb had only EPPK, but not other plakins, in KU-8 cell extract. In addition, anti-EPPK pAbs reacted clearly with lung and skin tissues of wild-type mouse, but no reactivity was observed in *Eppk*-knockout mouse. These results convincingly confirmed that pAbs to both human and mouse EPPKs did not cross-react with other plakin proteins.

However, we could not exclude completely the possibility that anti-EPPK autoantibodies in PNP sera might cross-react with other plakin proteins. Indeed, the positive reactivity of all PNP sera with EPPK RP might be due to cross-reactivity with other plakin proteins.

Nevertheless, we convincingly confirmed that sera from patients with PNP reacted clearly and specifically with EPPK in our IP-IB of KU-8 cell extract, which strongly suggested that sera from patients with PNP had autoantibodies to EPPK. In addition, statistical analyses strongly indicated that EPPK was related to BO development. These results adequately indicated that autoantibodies, which have specific or stronger reactivity with EPPK, were present in the majority of the PNP sera, and may cause BO in particular patients.

In conclusion, this study showed that EPPK is one of PNP autoantigens, and the presence of anti-EPPK antibodies is significantly related to the development of BO and higher mortality. Abnormal histopathological changes were found in lung tissues in mice injected with anti-EPPK pAb, indicating a pathogenic role of anti-EPPK antibodies in the development of BO. Therefore, detection of anti-EPPK antibodies may lead to more extensive treatments to avoid the development of fatal PNP-related BO.

MATERIALS AND METHODS

All studies followed guidelines of Medical Ethics Committees of Kurume University School of Medicine, and were conducted according to Declaration of Helsinki principles. Informed consents were written by all patients and control individuals.

Materials

All chemicals used for biochemical analyses were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO) or Nacalai Tesque (Kyoto, Japan). Details for preparation of anti-EPPK pAb and anti−mouse-EPPK pAb are described in Supplementary Materials and Methods online. Horseradish peroxidase-conjugated goat antibodies to human IgG, rabbit IgG, or mouse IgG for second antibodies in IB were obtained from Dako (Glostrup, Denmark).

This study used 48 PNP sera that were collected between 1997 and 2007 at the Department of Dermatology, Kurume University School of Medicine. Three PNP cases were treated in Kurume University Hospital and the rest were sent from other institutes. All patients with PNP showed typical clinical, histopathological, and immunopathological features. All 48 PNP sera reacted with the 210-kDa EPL and 190-kDa PPL by IB using normal human epidermal extract (Hashimoto, 2001). We also obtained 22 sera from European patients with PNP (16 from the Netherlands, 4 from Germany, and 1 each from Poland and Hungary). Sera were obtained from 20 patients each with pemphigus vulgaris, pemphigus foliaceus and BP as disease controls, and from 35 healthy volunteers as normal controls. All sera were stored at −30 °C or −80 °C, and aliquots with 0.1% sodium azide as a preservative were kept at 4 °C during experiments.

BALB/c adult mice (5-week-old) were purchased from Kyudo (Tosu, Japan). All operations with mice (breeding, anti−mouse-EPPK pAb injection and tissue collection) were ordered to Kyudo.

Preparation of human and mouse EPPK RPs and pAbs to them

Technical details are described in Supplementary Materials and Methods.

IHC and IF for EPPK

We performed IHC or IF of normal human skin and lung tissue, as well as KU-8 cells and NHSAE cells, using anti-EPPK pAbs. We also performed IF of skin and lung tissue taken from wild-type and *Eppk*-knockout mice. Technical details are described in Supplementary Materials and Methods.

IB of normal human epidermal extract, extracts of KU-8 cells and NHSAE cells, and human EPPK RP

We performed IB analyses of various substrates for detecting EPPK. Technical details are described in Supplementary Materials and Methods.

Adsorption of anti-EPPK pAb and anti−mouse-EPPK pAb with EPPK RPs

To confirm the specificity of anti-EPPK pAb or anti−mouse-EPPK pAb, we performed adsorption analysis using EPPK RPs. Technical details are described in Supplementary Materials and Methods.

IP-IB of KU-8 cell and NHSAE cell extracts

Technical details are described in Supplementary Materials and Methods.

IP-IB for PNP sera using KU-8 cell extract preabsorbed with anti-EPPK pAb, and combined IP-IB using antibodies specific to various plakin family proteins

Technical details are described in Supplementary Materials and Methods.

Injection experiments of anti−mouse-EPPK pAb injection into mice

Technical details are described in Supplementary Materials and Methods.

Dsg1 and Dsg3 ELISAs

ELISAs for Dsg1 and Dsg3 were performed according to standard procedures (MBL, Nagoya, Japan) (Billet et al., 2006).

Statistical analysis

Differences among qualitative results were statistically compared using the chi-square test and Bonferoni adjustment. Differences in quantitative parameters among different groups were assessed

using the Mann-Whitney test. All data are expressed as means ± standard deviation. All analyses were performed using SPSS (SPSS, Chicago, IL).

Amino acid sequence alignment of plakin family proteins

Multiple sequence alignment of plakin family was made with ClustalW (www.genome.jp/tools/clustalw) and MacBoxshade version 2.15 (Institute for Animal Health, Woking, UK).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at doi:10.1038/JID.2015.408.

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